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INDOLE METABOLISM. I.—THE COLORIMETRIC ESTIMATION OF INDOLE BY THE XANTHYDROL REACTION.

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INDOLE METABOLISM. I.—THE COLORIMETRIC ESTIMATION OF INDOLE BY THE XANTHYDROL REACTION.

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For the requirements of a study of the metabolism of indole in the animal body, it was found necessary to employ a method for the detection and estimation of indole that was more selective than the aldehyde condensation methods in current use, and more sensitive than the selective napthoquinone method introduced by Herter and Foster (1906). The method finally adopted is based on the production of a stable violet pigment when indole and xanthydrol react in acid solution. This reaction, reported independently by Fearon (1944) and by Arreguine (1944), has been used for the estimation of indole by Gordon and Mitchell (1949), who, however, do not give the details of the method employed. Zappacosta (1935) described a method for the determination of indole and scatole in serum using xanthydrol as a reagent. The compounds are isolated and converted into the 'dixanthyl' derivatives, dissolved in acetic and trichloracetic acids, and the resultant red blue colour compared with standards. The isolation and preparation of the dixanthyl derivatives, involving evaporations on a water bath, renders the method extremely liable to error.

The xanthydrol test, as described by Arreguine, involves the use of a strong mineral acid as a condensing agent, and is of low selectivity, a wide range of β-indole derivatives reacting, including tryptophan. Furthermore, under such conditions xanthydrol is converted into an oxonium ion, the bright yellow colour of which complicates subsequent colorimetric analysis. By employing glacial acetic acid only, as demonstrated by Fearon in 1943, the reaction becomes much more sensitive and selective. It is not, however, absolutely specific, the β-methyl derivative of indole, scatole, also reacts. Where scatole is present, the indole can be estimated, using the β-napthoquinone method of Herter and Foster.

The xanthydrol reagent reacts also with indole propionic and indole acetic acids to give a pink colour, but only in high concentration and on prolonged boiling. Some non-biological substances, notably unsubstituted barbituric acid, also react chromatically.

Procedure.

Reagents: (1) *Standard Indole Solution.* A stock solution is prepared by dissolving 100 mg. of recrystallized indole in 100 ml. of absolute ethanol. Kept in the dark, this solution appears to be stable indefinitely. Working standards are prepared by diluting aliquots of this solution with distilled water.

(2) *Glacial Acetic Acid free from Glyoxylic Acid.* The purity of the reagent can be tested by boiling 3 or 4 ml. with a few mg. of indole. If a pink colour develops, the acid must be purified by distillation and rejection of the first 20 per cent. of the distillate, which contains the glyoxylic acid. Stored in the dark, the acid retains its purity indefinitely.

(3) *Xanthydrol, 5 per cent., in Absolute Ethanol.* The solution retains its working activity for at least a week if stored at 0° C, but xanthydrol is unstable, both in solid form and in solution, and should be prepared in small batches, as required, by the method described by Werner (1923).

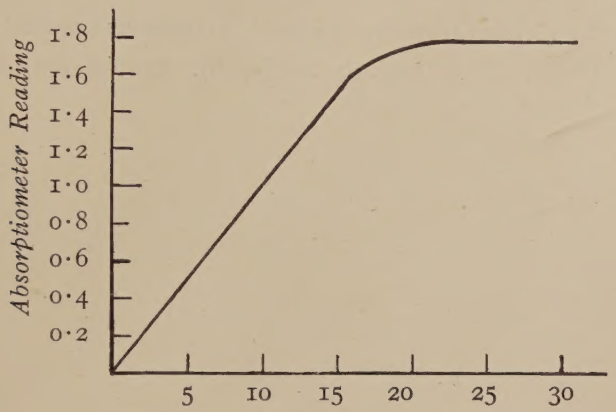
(4) *Preparation of Material for Analysis.* Although the reaction can be applied directly for estimation of indole in simple aqueous solutions or in dilute biological liquids, generally speaking, it is advisable to isolate indole before estimation to avoid the interfering effects of such common biological substances as non-volatile indole derivatives, urea, etc. Two methods are available for the isolation of indole: steam distillation and extraction with organic solvents. The latter method, rendered selective by the fact that indole may be extracted from acid or alkaline solution, is of use when distillation may be difficult owing to excessive foaming, but the ease of isolation of indole by steam distillation makes this the most convenient method. The distillation of indole is carried out under conditions which will vary with the nature of the indole-containing material. The amount taken and the amount of distillate collected will depend upon the original concentration of indole. The distillation may be performed at different pH values to prevent the passage of other volatile substances. High acid concentrations must be avoided, as the indole will polymerize under these conditions. In the course of the present investigations, indole was distilled from liver tissue culture. The distillation was carried out at pH 7.5 in buffered solution, and 100 ml. of distillate were collected. Blank experiments show that recovery figures for the distillation were almost theoretical. Solutions containing 1,3 5,7 10,15 mg. of indole were distilled. In each case 100 ml. of distillate were collected. Standards of 100 ml. containing the same amount of indole as distilled were made up, and each solution compared with the corresponding one by means of a Spekker absorptiometer.

Mg. Indole	Absorptiometer Reading		Recovery
	Distillate	Standard	
1	0.182	0.182	100%
3	0.415	0.425	97%
5	0.68	0.68	100%
7	0.92	0.92	100%
10	1.22	1.24	98%
15	1.6	1.6	100%

(5) *Method of Analysis.* One or two ml. of the indole solution or distillate are taken, and ten drops of a fresh solution of xanthydrol in ethanol are added. Standards for comparison are prepared by diluting aliquots of the standard indole

solution to 100 ml. with water, taking one ml. of the resultant solution and treating it as described. Three, seven and ten ml. of the standard indole solution diluted to 100 ml. were found to provide a suitable range of standards for comparison. The tubes containing the unknowns and standards are made up to 8 ml. with glacial acetic acid and are then placed in a boiling water bath for ten minutes to develop the colour, removed, and allowed to cool. The resulting coloured solutions are compared in a colorimeter, or preferably in an absorptiometer, in which case a calibration curve can be used.

Calibration Curve.—The relationship between light-absorption and pigment-concentration, as shown in the curve, is linear within a range that depends on the concentration of indole and the concentration of xanthydrol. The abscissae represent the concentrations of the original solutions, 1 ml. of which was taken and used for the estimation. For concentrations greater than 20 mg. per 100 ml. of solution, the curve flattens. Hence for estimations beyond this point, the original indole solutions must be diluted appropriately. For most purposes, the 5% solution



of xanthydrol has been found to be convenient. The deterioration of the xanthydrol solutions necessitates a fresh calibration curve being prepared for each series of estimations. For calibration it is sufficient to determine three points on the straight line from three standards usually of concentrations 3, 7 and 10 mg. indole per 100 ml. These also provide a suitable range of standards for comparison in ordinary colorimeters. The maximum aliquot of indole which can be estimated under the specified conditions is 5 micrograms.

The Solubility of Indole in Water. No reference to the solubility of indole in water could be found in the literature. The xanthydrol method was used to determine its solubility at 18° C, where it was found to be 220 mg. indole per 100 ml. of water, and at 38° C, where it was found to be 400 mg. indole per 100 ml. of water.

SUMMARY.

A colorimetric method for the estimation of indole, based on its condensation with xanthydrol in glacial acetic acid, is described.

This investigation was carried out in the Department of Biochemistry, Trinity College, Dublin, and one of us (J. A. D.) is indebted to the Medical Research Council of Ireland for a grant to enable this work to be done.

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